New approach to increase information content in polarised light microscopy of skeletal and dental tissues.

Alan Boyde[1], Alessandro Felder[2] & David Mills[1]
[1]QMUL & [2]RVC

Many thanks to CAIRN RESEARCH

Abstract
We introduce new and extremely simple ways of colourising and improving the information content of polarised light microscopy (PLM) images of bony and other tissues. Any polarising microscope in which one can rotate linear polarising filters and fitted with a digital camera can be used to obtain meaningful and interpretable results from suitable thin plane parallel sections.

In conventional PLM using linearly polarised light (LPL), birefringent structures appear brightest if they lie both in the plane of the section and at 45º to the axes of the crossed polarising filter elements, and dark if they lie parallel to either polariser or analyser, or perpendicular to the plane of section. This situation prevents us from comprehending the whole scene at once, because nothing can be seen in the dark sectors of the 'Maltese cross'.

This problem may be partially overcome by using circularly polarised light (CPL: images at bottom of this column). However, as we show here, it may also be simply solved by combining at least three - but any greater number of - monochrome PLM images.

In practical terms, what one can determine by PLM study of bone is the presence of oriented collagen and its orientation – but there are problems. Areas of bone tissue which appear black between crossed polarisers may do so because (a) they would appear black anyway (e.g., due to poor infiltration with the mounting medium): or (b) they do nor contain any birefringent material: or (c) the birefringent material is parallel with the optic axis [perpendicular to the plane of section]; or (d) is parallel with the polariser of the analyser. Collagen which is parallel to the section plane and in the 45° or 135° sectors [taking 0°-180° as the polariser and 90°-270° as the analyser vibration directions] appears maximally bright.

These effects together give rise to the 'Maltese cross' appearance of cross sectioned osteons and the alternate brighter and darker lamellae in the 45°/135° sectors.

Immature 'woven' bone contains some collagen fibre bundles large enough to be resolved by conventional LM, but only those at near 45° to the polarising filters are seen. These look like the warp and the woof of a woven fabric, but the structure is a feltwork.

Further problems for interpreting the PLM image arise from the effect of the degree of mineralisation of the tissue and the need to have plane parallel sections – i.e., of uniform thickness, which cannot be simply achieved for spongy bone – and that the sections need to be 'cleared', i.e., optically transparent and best invisible if not examined in polarised light.

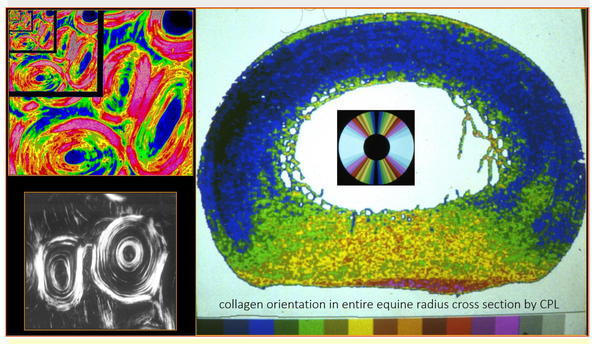
Coloured images of bone sections are produced when using crossed polarisers with a full wave retardation plate (sensitive tint plate: images at top of next column). Under these conditions, regions which would appear dark under standard LPL appear pink/mauve and still give no information, whilst collagen in one sector (45° or 135°) appears yellow/orange, and blue in the other.

In the CPL arrangement [images below], we use two additional quarter wavelength retardation plates at 45° and 135°. Collagen perpendicular to the section plane appears dark, but there is no Maltese cross, and we can see alternate light and dark lamellae in any azimuth. Thus the signal due to collagen fibre orientation is constant irrespective of its axis in the plane of section. The intensity for one orientation is related to the section thickness, and we must therefore use plane parallel sections. If we have plane parallel sections of compact bone we can pseudo-colour the resultant CPL bone images to produce very useful collagen orientation maps. These, however, do not differentiate between various possible in-plane orientations of the collagen.

The present report concerns new, very simple methods for extending the usefulness of PLM in this regard. The driving motivation was to exploit the high quality, thin, undecalcified sections produced by laser ablation microtomy which enable exact correlation with backscattered electron scanning electron microscopy imaging. We exploit digital processing to pack in more useful 3D LPL information in an image, yet hopefully in a way that may be easily understood and interpreted.

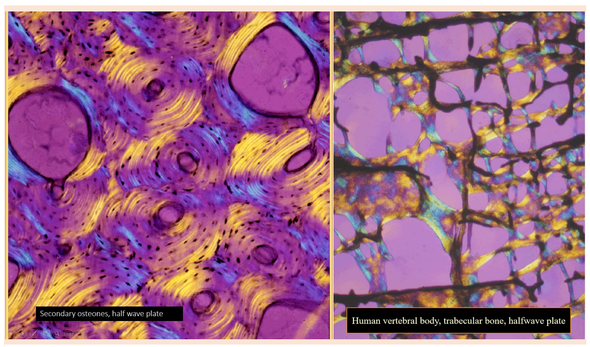
What we describe here can be easily repeated in any PLM in which the polarising filters can be rotated around a stationary sample, but we have automated the process of rotating the polarising and analysing filters together for multiple rotations at for example, 3°, 7.5°, 10°, 15° or 30° intervals, normally through a range of 90°, with LPL images recorded at each orientation. We thank Cairn Research, Faversham for developing the PLM automation.

As a common example for bone, we record six images with crossed linearly polarising filters at 15° rotation intervals and merge them using ImageJ. We can start where we like, but we use them in the colour circular sequence Red, Yellow, Green, Cyan, Blue, Magenta. Colour shows the orientation within the section plane, with 4 repeat cycles in 360º. Brightness is proportional to the cosine of the strike angle with respect to section plane, being brightest in plane, and black when perpendicular to that plane, i.e., parallel to the optic axis.

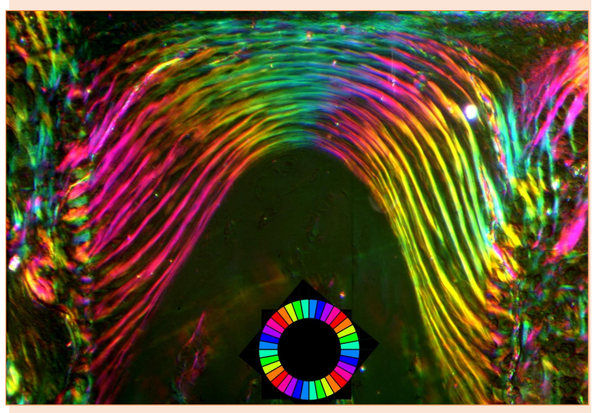


CPL = slice examined between crossed circularly polarising filters
- polar / 45° ¼λ plate/ bone section / 135° ¼λ plate / analyser
- All collagen parallel to section plane appears bright
- Collagen perpendicular to section plane appears dark
- No Maltese cross: alternate light dark lamellae in any azimuth
- signal due to collagen fibre orientation is constant irrespective of axis in the plane of section.
- Intensity linearly related to section thickness for one orientation.

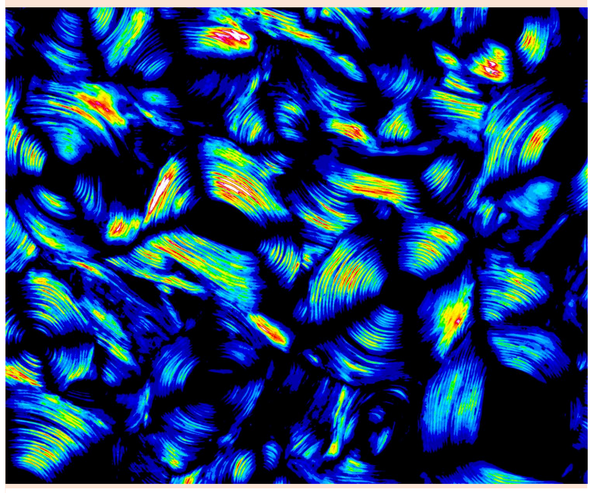
The quantitative study of the orientation of collagen in compact bone slices. Boyde A, Riggs CM. Bone 1990 11:35-39.



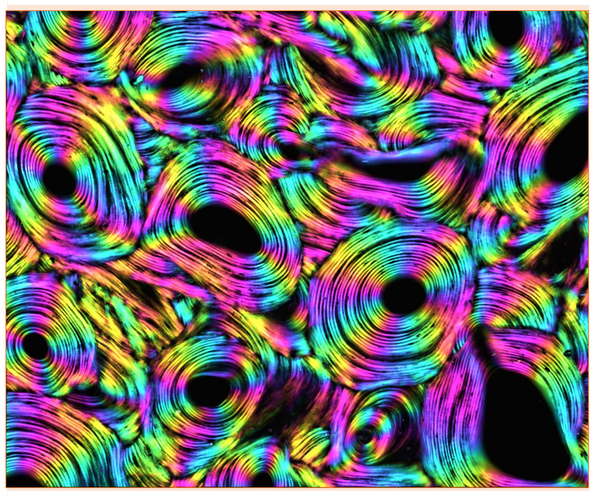
Use of a half-wave plate with crossed polars produces pretty pictures which cannot be quantified. Very common in the literature. Sample must be plane-parallel to make any real sense of the data.



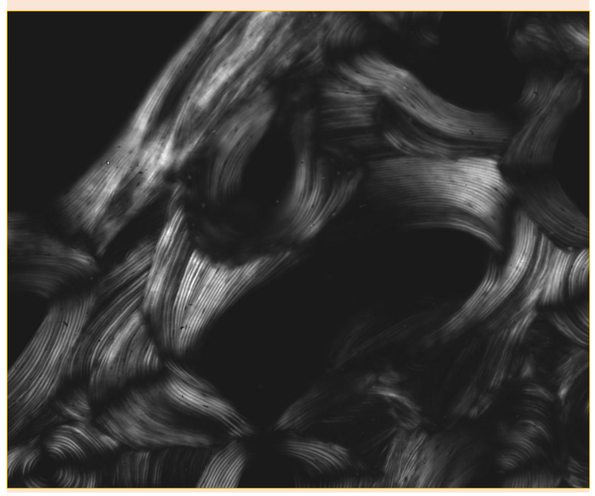
From 'New method for quantitative polarised light microscopy of laser-ablation machined sections of bones and joints', Bone Research Society poster 2017, which described the use of 3 images at 30° rotations of crossed polars. Talked to Jeremy Graham, Cairn Research, Faversham at MMC 2017 and they built the PLM automation system we now use. THANKS !



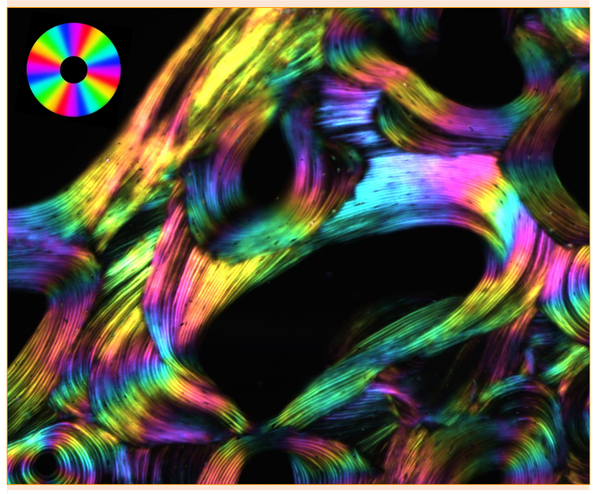
One monochrome PLM image of a set, pseudocoloured with ImageJ 16cols LUT. Human Femur TS ground section. Field width 1220 µm.



PLM image set with 15° rotations merged to give pseudocolours in the sequence Red Yellow Green Cyan Blue Magenta. Human Femur TS ground section 10X. Field width 1220 µm

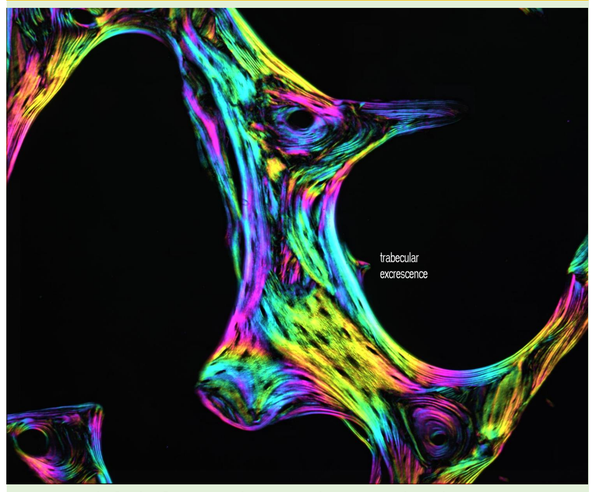


One monochrome PLM image of a set. Human Femur TS ground section, 10X objective. Field width 1220 µm

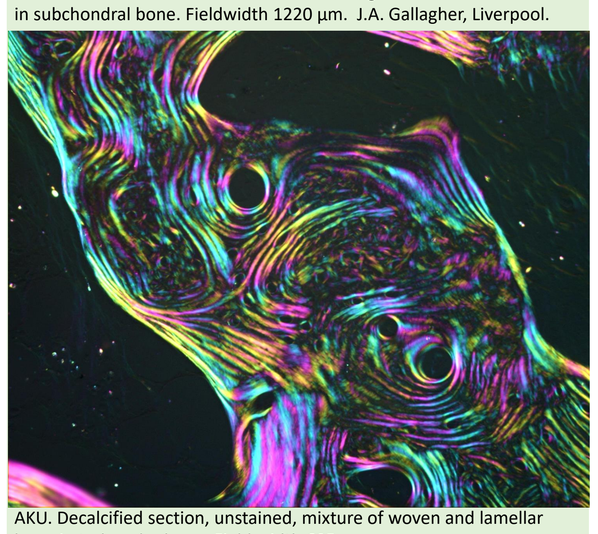


PLM image set with 15° rotations merged to give pseudocolours in the sequence Red Yellow Green Cyan Blue Magenta. Human Femur TS ground section 10X. Field width 1220 µm.

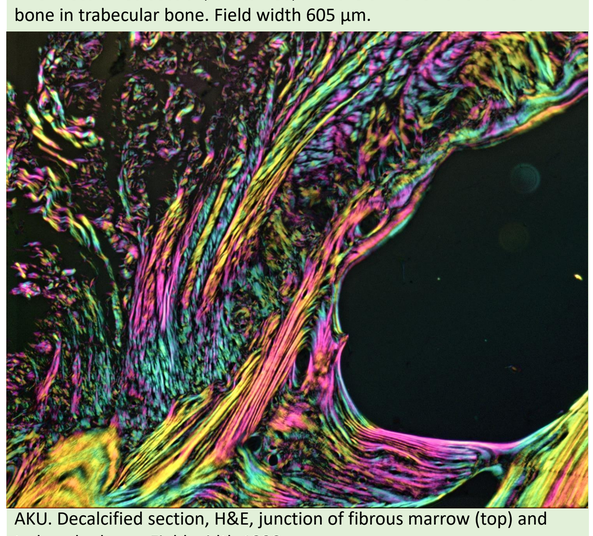
OSTEOARTHRITIC SUBCHONDRAL BONE MARROW HISTOLOGY. LESSONS FROM CHANGES IN ALKAPTONURIA (AKU).



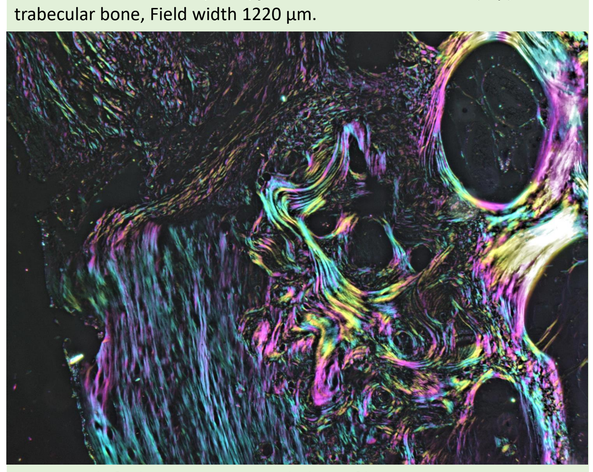
AKU. Decalcified H&E stained section showing trabecular excrescence in subchondral bone. Fieldwidth 1220 µm. J.A. Gallagher, Liverpool.



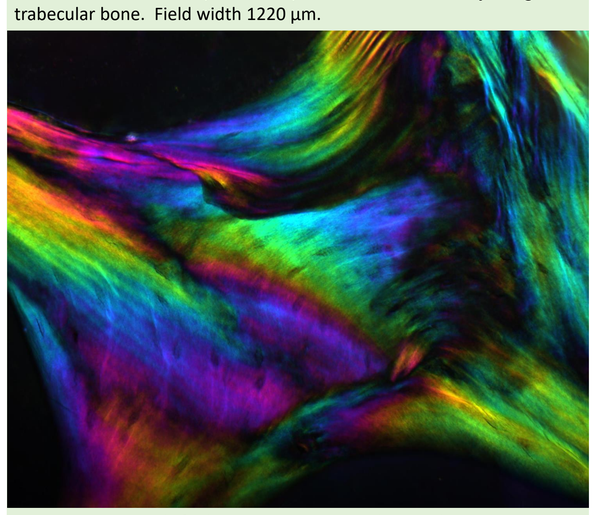
AKU. Decalcified section, unstained, mixture of woven and lamellar bone in trabecular bone. Field width 605 µm.



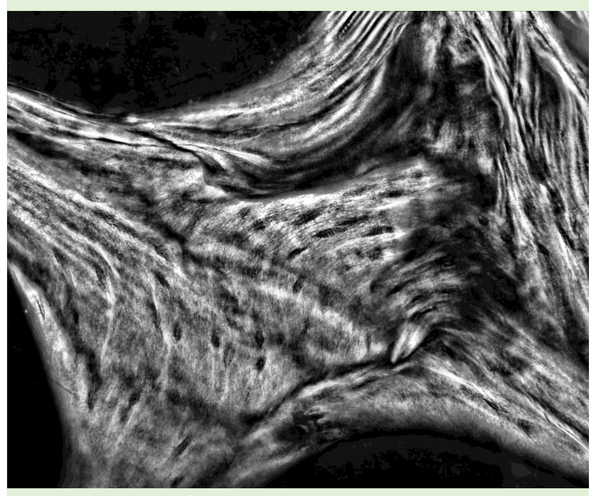
AKU. Decalcified section, H&E, junction of fibrous marrow (top) and trabecular bone, Field width 1220 µm.



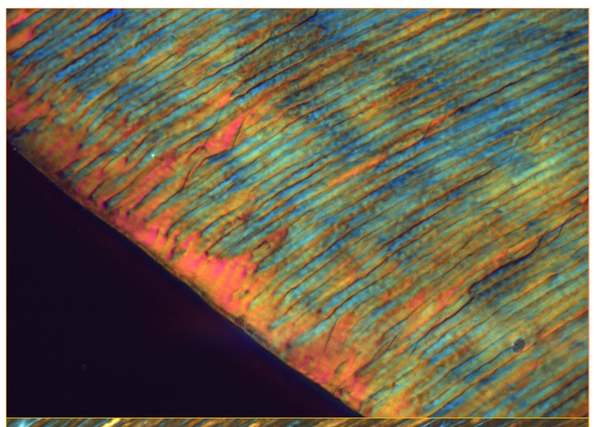
AKU. Decalcified section, unstained, dense fibrous marrow joining trabecular bone. Field width 1220 µm.



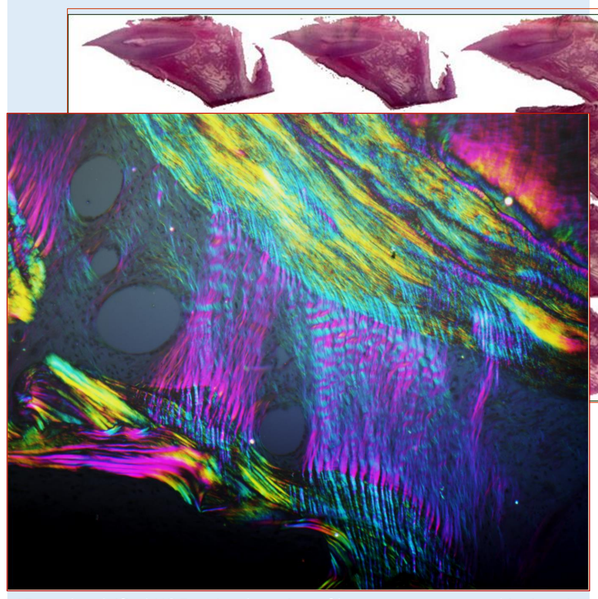
AKU. 10 µm undecalcified section prepared by laser ablation microtomy (J Microscopy 2018). 3 images at 30° to RGB. Fieldwidth 302 µm.



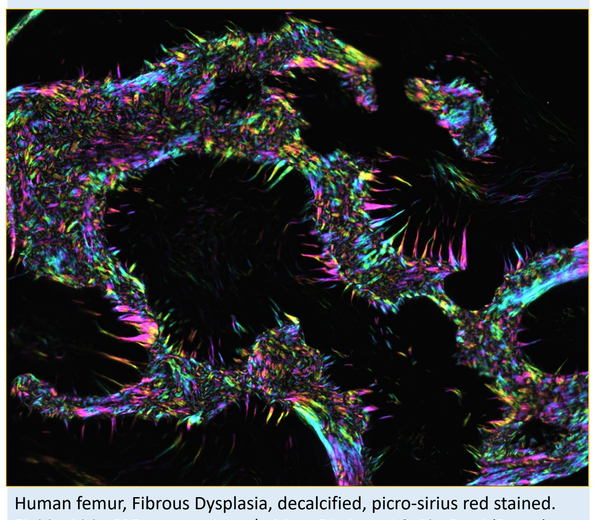
AKU. 10 µm undecalcified section prepared by laser ablation microtomy. 18 images at 5°, **maximum intensity.** Fieldwidth 302 µm.



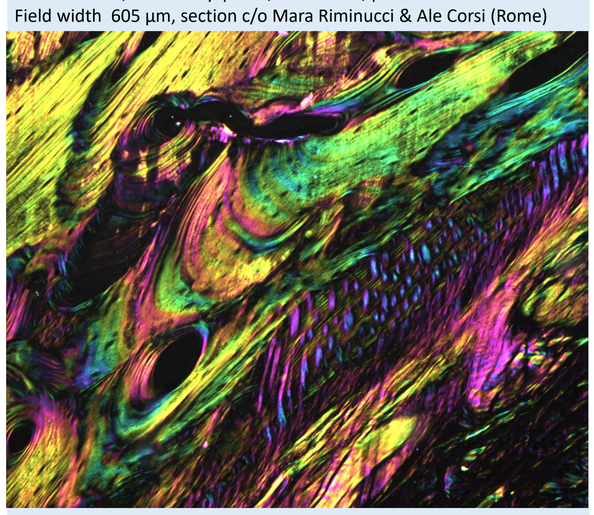
8µm Laser Ablation Microtomy section of David Mills's upper third molar ENAMEL. Images processed from set of 15. Field width 302 µm.



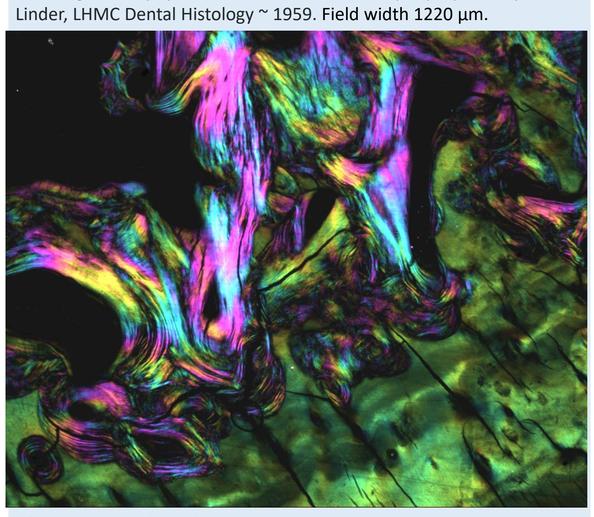
15µm decalcified H&E stained section of human upper central incisor *in situ.* Slide made by B Gottlieb, Vienna, ~ 1923.



Human femur, Fibrous Dysplasia, decalcified, picro-sirius red stained. Field width 605 µm, section c/o Mara Riminucci & Ale Corsi (Rome)



Jaw condyle, cortex with dense fibrous periosteum, bottom right, inserting as Sharpey Fibres. Decalcified, H&E, 10µm prepared by JE Linder, LHMC Dental Histology ~ 1959. Field width 1220 µm.



10µm laser ablation microtomy section human knee, osteoarthritis. Articular calcified cartilage at lower right. Field width 1220 µm. Collaboration with Nidhi Sofat SGUL.